Chi-Kuang Wen *Editor*

Ethylene in Plants

Springer

Chapter 13
Research Tool: Ethylene Preparation: Treatment with Ethylene and Its Replacements

Mark Tucker and Chi-Kuang Wen

Abstract Ethylene gas is an important plant hormone that can be chemically prepared or biologically synthesized by microbes and plants. The gas can be commercially obtained in a pressurized gas cylinder or chemically prepared with necessary purifications. Laboratories that wish to perform experiments involving ethylene treatment need a convenient setup for ethylene preparation and delivery. When the use of a pressurized ethylene gas cylinder is not feasible, an ethylene response can be initiated in the plant or plant organs by treating the plant or organ with an aqueous solution of the natural plant precursor to ethylene, 1-aminocyclopropane-1-carboxylic (ACC), or 2,4-dichlorophenoxyacetic acid (ethephon), which decomposes slowly to make ethylene at a pH above 4.0. However, the release of ethylene for these applications is dynamic and not experimentally controllable, and, moreover, the replacement may produce unwanted side effects that can affect data interpretation. Therefore, a direct ethylene treatment is often favorable over the replacement. An alternative to a pressurized tank of ethylene is the chemical synthesis of ethylene by ethanol dehydration or ethephon decomposition, with a specific setup to collect the produced ethylene. This chapter discusses the advantages and disadvantages of direct ethylene treatment using ethylene gas or a replacement. Also discussed are the underlying chemical and biochemical reactions for ethylene production, and the setup for ethylene treatment in a closed system or a flow-through system. The goal is to provide readers with the necessary tools for ethylene treatment with easily accessed laboratory devices.

M. Tucker (✉)
Soybean Genomics and Improvement Lab, USDA/ARS, Bldg 006, BARC-West, 10300 Baltimore Ave, Beltsville, MD 20705, USA
e-mail: Mark.Tucker@ARS.USDA.GOV

C.-K. Wen
National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
e-mail: qgwen@sibs.ac.cn

C.-K. Wen (ed.), *Ethylene in Plants*, DOI 10.1007/978-94-017-9484-8_13

Keywords Ethylene · 1-Aminocyclopropane-1-carboxylic acid (ACC) · 2,4-Dichlorophenoxyacetic acid (ethephon)

13.1 Introduction

Ethylene is a gaseous plant hormone regulating many aspects of plant growth and development. For research purposes, there are advantages and disadvantages to working with a gaseous hormone. Because it is a gas, it is quickly and easily disseminated inside a closed chamber. Commercially, fruit (e.g., bananas, tomatoes, avocado) can be packed into a large chamber and cheaply and efficiently exposed to ethylene to induce ripening (Abeles et al. 1992). Biological experiments involving ethylene treatment require a pressurized ethylene gas cylinder as an ethylene source and necessary devices for handling and delivering the gas. Unlike other plant hormones that can be delivered and treated as a solution, the gas cylinder and necessary devices for ethylene treatment may not be readily available in laboratories that only occasionally perform the treatment. Nonetheless, unlike most other plant hormones, which have specific mechanisms for uptake, transport and metabolism, ethylene diffuses rapidly into the plant through stomata and is water and lipid soluble, which allows it to readily move across cell membranes with no specific transport mechanism (Abeles et al. 1992). Experimentally, because multicellular plants have interconnecting gas space to every cell and diffusion through gas is 10,000-fold faster in air than water, this means that, although the plant may synthesize more ethylene when exposed to ethylene, the concentration you present on the outside of the plant is the minimum concentration that a cell on the inside of the plant perceives at its surface. The concentration at the surface of a cell for other plant hormones (e.g., IAA, BA, ABA, JA) is not so easily known. However, there are experimental circumstances where a gaseous treatment is not feasible. In this case, a soluble replacement (e.g., ACC or ethephon) can be used to induce an ethylene response in the plant.

The ethylene biosynthesis pathway in higher plants begins with the amino acid methionine, which is converted to S-adenosyl-L-methionine (SAM). SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is the immediate ethylene biosynthesis precursor and is converted to ethylene and cyanide on oxidation by the ethylene-forming enzyme ACC oxidase (ACO) (Adams and Yang 1979; Peiser et al. 1984; Kende 1993; Yoon and Kieber 2013). Cyanide is a toxic byproduct of ethylene production with ACC oxidation and is removed immediately by the conversion to β-cyanoalanine and asparagines (Miller and Conn 1980). Of note, cynoformate is an intermediate product formed during the reaction to shuttle away the toxic cyanide from the active site of ACO, such that the iron-containing ACO is protected from the cyanide deactivation (Murphy et al. 2014). The formation of ACC is the rate-limiting step for ethylene production, and an elevated ACC level results in higher ethylene evolution. Therefore, ACC is

widely used as an ethylene replacement. However, ACC is consumed shortly after application (Zhang et al. 2010; Zhang and Wen 2010; Lavee and Martin 1981); replacing ethylene with ACC may not be ideal for quantitative experiments requiring a long response window.

Another replacement for ethylene is ethephon (also called Ethrel). Under alkaline conditions, ethephon (2,4-dichlorophenoxyacetic acid) decomposes to ethylene, phosphate, and chloride (Lavee and Martin 1981; Yang 1969; Biddle et al. 1976; Zhang and Wen 2010). Aqueous ethephon solutions are widely used as a replacement for ethylene treatment. The uptake and decomposition of ethephon *in planta* is unclear and not experimentally controllable, and the decomposition products phosphate and chloride that produce a low pH condition may have adverse effects on many aspects of plant growth and physiological processes (Reid et al. 1980; Goudey et al. 1987; Southwick et al. 1986; Zhang and Wen 2010). Thus, using ethephon as a replacement for ethylene treatment is not ideal for quantitative experiments requiring a long response window, and unwanted effects with the strong acid phosphate and chloride produced by ethephon decomposition must be evaluated.

For a plant laboratory that may not want to invest in a pressurized tank of ethylene and are concerned about applying ethephon directly on the plant, ethylene can be prepared by chemical decomposition of ethephon or complete ethanol dehydration. The ethylene released in these systems can then be used in a closed system to induce an ethylene response in the plant.

This chapter describes the use of ethylene replacements and possible unwanted effects associated with the replacements, the chemical preparation of ethylene with use of standard laboratory equipment, the delivery and transfer of the gas, the setup for an airtight chamber for ethylene treatment, and a flow-through system for experiments requiring a stable ethylene concentration environment. Researchers with different experimental needs may choose appropriate approaches for ethylene treatment.

13.2 Ethylene Treatment with the Use of Replacements

ACC and ethephon are the two most widely used replacements for experiments involving ethylene treatment. Both are solid, water soluble, and thus easily prepared. ACC is an intermediate product of ethylene biosynthesis and ethephon is not; the former can be oxidized to produce ethylene by ACC oxidase, whereas mechanisms for the absorption and decomposition for the latter *in planta* is unknown. Although convenient, both have limitations and drawbacks when used to replace ethylene. Ethylene is released within a short response window by the replacements, and the release is dynamic and uncontrollable; therefore, the treatment can be qualitative but not quantitative and thus the result is less reproducible. Nevertheless, the replacements may have other advantages. Several factors should be considered for proper experimental design with ethylene replacements.

13.2.1 ACC as an Ethylene Replacement

ACC, $C_4H_7NO_2$, with a molecular mass 101.1 g $mole^{-1}$, is a solid and is readily dissolved in water. The concentration and amount of ACC to be used is dependent upon the desired amount of ethylene production, which is not necessarily constant throughout the treatment window (Lavee and Martin 1981; Zhang and Wen 2010; Zhang et al. 2010). A sufficient supply of ACC is essential to ensure a prolonged ethylene exposure. Otherwise, the ethylene released by ACC oxidation will decrease over time because of ACC consumption. For experiments involving ethylene effects on inhibiting seedling hypocotyl growth, the seeds are germinated and grown on a relatively large volume of Murashige and Skoog (MS) medium in agar with a large amount of ACC at the necessary concentrations so that ethylene biosynthesis is sustained through a desired growth period. *ETHYLENE RESPONSE FACTOR1* (*ERF1*) is a primary target of the ethylene signal, and its induction is directed by the transcription factor ETHYLENE INSENSITIVE3 (Solano et al. 1998). The expression of *ERF1* is linked to the degree of the ethylene response and thus is a form of quantification for the ethylene response. *Arabidopsis* seedlings grown on MS-containing agar supplemented with ACC show nearly identical *ERF1* expression as those treated with a saturating concentration of ethylene, e.g., 10 μL L^{-1}. In contrast, *Arabidopsis* plants treated with a foliar spray of ACC do not show reproducible *ERF1* expression (Zhang and Wen 2010). For hypocotyl growth experiments, the seedlings in this short-term treatment only consume a small fraction of the supplemented ACC, and therefore, the conversion of ACC to ethylene is sufficient and sustained for the necessary time frame. When ACC was applied as a foliar spray, the amount of ACC absorbed by individual plants may have varied and consumed quickly. Thus, a foliar spray may be ideal for experiments requiring a short response window. Prolonged ethylene exposure would require periodic sprays of ACC over the entire experiment. Nevertheless, the amount of ACC sprayed on an individual plant may vary, and the ethylene produced also varies; thus, foliar sprays of ACC may be less reproducible.

Although ACC has been used to replace ethylene treatment for analysis of seedling growth inhibition, when non-maximal concentrations of ethylene and ACC are used, the effects on *Arabidopsis* growth inhibition differ slightly. Over a wide concentration range, the ethylene dose–response shows a concave curve, whereas the ACC dose–response curve is convex, with 50 % growth inhibition for 0.1–0.2 μL L^{-1} (ethylene) and 0.5 μM (ACC) (Zhang and Wen 2010). Aminoethoxyvinylglycine (AVG) is a potent ethylene biosynthesis inhibitor. Ethylene treatment with AVG supplementation does not affect *Arabidopsis* seedling growth inhibition over a wide ethylene concentration range. In contrast, with ACC treatment to replace ethylene, with AVG supplementation, seedling growth inhibition is in part alleviated over a wide range of ACC concentration. Thus, without AVG to prevent endogenous ethylene production, ACC treatment may trigger an increase in endogenous ethylene production, whereas treatment with ethylene does not.

Several factors should be considered when ACC is used to replace ethylene treatment. In higher plants, the amino acid methionine is adenylated to form SAM, an important methyl donor involved in many biological processes and the biosynthesis of polyamines and ACC (Pommerrenig et al. 2011). ACC is the immediate precursor for ethylene biosynthesis on oxidation by ACOs. Thus, replacing ethylene treatment with ACC requires sufficient oxygen to ensure the ethylene production reaction. ACC may not be efficiently converted to ethylene for experiments performed under hypoxia (or oxygen shortage). Thus, for an ACC treatment performed in small closed vials or containers, in which oxygen is used for respiration, oxygen availability may be insufficient to support prolonged growth, and the ACC oxidation reaction attenuated. No matter, hypoxia in itself can impose adverse effects on plant growth. For experiments carried out in a closed system, with ACC used to replace ethylene, sufficient oxygen is needed to support ACC oxidation and plant growth.

Of note, lower plants also produce ethylene, and the gas has biological effects on many aspects of growth and development in ferns. The unicellular spores of the fern *Onoclea sensibilis* can germinate in darkness and produce 2 cells by cell division, and the initial division is inhibited by ethylene as low as 0.1 μL L^{-1} (Fisher and Shropshire 1979; Edwards 1977). However, lower plants do not seem to use ACC as a precursor for ethylene biosynthesis. Although ACC is present in ferns, ACC treatment does not increase ethylene evolution. Treatment with aminoethoxyvinylglycine and α-aminoisobutyricacid, the inhibitors of the ethylene-forming enzymes ACS and ACO, respectively, does not inhibit ethylene production in the semi-aquatic ferns *Regnellidium diphyllum* Lindm. and *Marsilea quadrifolia* L. (Chernys and Kende 1996). When these plants were treated with radioactive [^{14}C]-ACC, the ACC was readily taken up and decarboxylated by the fern *R. diphyllum* and the liverwort *Riella helicophylla*, but the [^{14}C]-ethylene was not released (Osborne et al. 1996). Thus, ACC is not a replacement for ethylene for experiments involving lower plants.

In higher plants, ACC can be malonylated to form N-malonyl-ACC (MACC) by ACC N-malonyltransferase with malonyl-CoA used as the malonyl donor (Peiser and Fa Yang 1998; Finlayson et al. 1991; Martin et al. 1995; Martin and Saftner 1995). MACC is not normally converted back to ACC for ethylene biosynthesis. The conversion of ACC to MACC is probably a mechanism to consume excess ACC to prevent excess ethylene production (Hoffman et al. 1983). When an excess of ACC is present in the plant, a certain fraction of ACC may be converted to MACC, which would accelerate ACC consumption.

In addition to ethylene, cyanoformic acid is formed on the oxidation of ACC by ACO, and cyanoformic acid is spontaneously degraded into cyanide and carbon dioxide (Peiser et al. 1984; Murphy et al. 2014; Adams and Yang 1979; Kende 1993). Cyanide is toxic and can be metabolized to form β-cyanoalanine and asparagine. Recent studies suggest that cyanide formed by ACC oxidation may have a role in rice (*Oryza sativa*) resistance to the blast fungus *Magnaporthe oryzae*. Treatment with ethylene alone has little effect on rice resistance to the blast fungus, whereas potassium cyanide (KCN) and ACC each confers the resistance.

Salicylhydroxamic acid (SHAM) is an inhibitor of cyanide-resistant respiration (Seo et al. 2011). The mycelium growth of *M. oryza* on potato dextrose agar (PDA) supplemented with KCN and SHAM was substantially inhibited, whereas KNC and SHAM alone had a minor effect on the fungal growth. The synergistic effect indicates that the prevention of cyanide-sensitive and cyanide-insensitive respiration is fatal to the fungal growth. However, it should be noted that the amount of cyanide and ACC used in the experiment was relatively high (0.5–1 mM for KCN and 0.5 mM for ACC), that may not necessarily occur *in planta*. Of note, although ACC as an ethylene replacement has limited use, ACC is an ideal replacement for ethylene treatment in experiments when treatment with ethylene is technically difficult. For instance, ACC but not ethylene treatment can be used to observe the effects of ethylene at the subcellular level in live cells by microscopy.

13.2.2 Ethephon as an Ethylene Replacement

Ethephon, $C_2H_6ClO_3P$, with molecular mass 144.5 g mole^{-1}, is a solid and is readily soluble in water (123.9 g 0.1 L^{-1} at 23 °C). The amount of ethephon needed for ethylene replacement can be determined experimentally. Ethephon is a dibasic acid (pKa1 = 2.24 and pKa2 = 6.97 at 25 °C), and commercially available ethephon solutions are acidic (pH about 2.3). Ethephon is in the form of monoanion at low pH and dianion at high pH. About 10 and 90 % of ethephon is in the dianion form at pH 6 and 8, respectively. Above pH 9, ethephon is 100 % in the dianion form, which is the form that undergoes decomposition into ethylene (Reid et al. 1980; Biddle et al. 1976; Yang 1969):

$$ClCH_2CH_2PO_3^{2-} + H_2O \rightarrow Cl^{-1} + C_2H_4 + H_2PO_4^-$$

The foliar application of aqueous ethephon solution has been widely used in agriculture. Lodging results in yield loss in tall cereals, such as barley and corn, and can be reduced with ethephon treatment to promote yield increases (Dahnous et al. 1982; Norberg et al. 1988). Ethephon treatment also increases the tiller number, millable canes, and yield of sugar cane (Li and Solomon 2003).

Foliar sprays or ethephon in hydroponic culture has been used to replace ethylene treatment in biological experiments. The release of ethylene from ethephon is dependent on both the pH and temperature of the aqueous environment. Thus, the buffering capacity of the solution the ethephon is dissolved in can have profound effects on the rate of ethylene released. Moreover, ethephon itself can contribute to buffering capacity when the treatment solution has weak or little buffer capacity. In weakly buffered solutions, the release of ethylene will decrease as the pH of the solution decreases as a result of ethephon decomposition. Also important to the efficacy of ethephon treatment is the uptake and movement of ethephon in the plant. Thus, complications with buffering capacity and movement of ethephon in the plant

require that appropriate treatment conditions be empirically determined. This limits its utility for research but has been very useful for well-characterized commercial applications.

Acids produced by ethephon may have other effects that are coupled with or not induced by ethylene and also promote ethylene biosynthesis to induce ethylene responses (Goudey et al. 1987; Reid et al. 1980; Zhang and Wen 2010). Of note, the amount of ethylene produced by ethephon decomposition is undetermined and may be dynamic over a response window; the combined effects of ethylene and the produced acids complicate the treatment, and the interpretation of the experimental data is difficult.

13.3 Chemical Preparations of Ethylene

Ethylene is widely used in the chemical industry and can be mass produced in the petrochemical industry by steam-cracking hydrocarbons; the procedure is complicated, energy intensive, and requires facilities that are not affordable for most laboratories to synthesize the gas. Ethylene and ethanol are interchangeable by hydration/dehydration reaction and ethanol dehydration with catalysts such as sulfuric acid, and aluminum oxide produces ethylene. The production of ethylene with industrial approaches may not be ideal for laboratories without the facilities to perform the reaction and purification. Nevertheless, ethylene can be produced by ethephon decomposition under mild conditions. Plant laboratories can easily set up ethylene production with the use of ethephon.

13.3.1 Ethanol Dehydration for Ethylene Production

Cracking ethanol to give ethylene and water can be used to produce ethylene by removal of the hydroxyl (–OH) group and hydrogen atom from the second carbon in the chain. With the use of acid as a catalyst for ethanol dehydration, the hydroxyl group is protonated by an acid and leaves as a water molecule; the methyl group of ethanol is then deprotonated by the conjugated base of the catalyst, and the hydrocarbon rearranges into ethylene. The reaction is zero-order and endothermic occurring at elevated temperature (180–500 °C) that shifts the equilibrium toward ethylene production. Of note, reactions to form diethyl ether or acetaldehyde are favored outside the temperature range, and byproducts are generated (Zhang and Yu 2013). Ethanol can also be oxidized by concentrated sulfuric acid to carbon dioxide and the acid reduced to sulfur dioxide at the same time. The ethanol dehydration by concentrated sulfuric acid is dangerous in a regular laboratory, and the produced ethylene contains byproducts that need to be removed before biological treatment to avoid unwanted effects.

Various solid acid catalysts such as zeaolites and silica-alumina have been used for ethanol dehydration to produce ethylene in industry (Takahara et al. 2005). γ-alumina (Al_2O_3) is an alternative catalyst for industrial ethanol dehydration; the ethylene yield is relatively low (80 %) and contains byproducts, and the reaction temperature is high (450 °C). With various modifications, γ-alumina-catalyzed ethanol conversion has been improved with a higher ethylene selectivity (Fan et al. 2012). Nevertheless, the reaction needs high temperature, and the produced ethylene must be purified to remove byproducts. In addition to γ-alumina, several nanoscale catalysts have been developed for ethanol dehydration, with relatively high conversion rate and ethylene selectivity at lower temperature (as low as 220 °C).

Although the production of a small amount of ethylene for a biological treatment does not have to consider conversion efficiency and cost-effectiveness, the ethylene produced by ethanol dehydration must be purified to prevent unwanted effects exerted by the byproducts. With a reaction taking place at high temperature, with various byproducts, ethanol dehydration can be dangerous and not a favored approach for laboratories that do not have the facilities for the reaction and purification.

13.3.2 Ethylene Production by Ethephon Decomposition

Ethephon has been widely used as a replacement for ethylene treatment. The chemical reaction that takes place for ethephon to produce ethylene is described above (Sect. 13.2.2). Providing a constant reaction condition that favors the decomposition of ethephon facilitates the production of the ethylene gas that can be used directly for an ethylene treatment without purification. Ethephon decomposition is favored under alkaline conditions. At pH > 9.0, the chemical is nearly completely in the dianion form, which decomposes readily into ethylene (Biddle et al. 1976; Yang 1969). Of note, ethephon decomposition produces acid compounds, phosphate and chloride, that will reduce the pH of the solution and slow the decomposition reaction (Sect. 13.2.2). Thus, to ensure constant decomposition, a strong buffer capacity is needed to maintain the reaction at a high pH. Because the products of the decomposition are ethylene and acids and the latter remain in the solution, the ethylene gas produced can be easily collected for use without the need for purification.

Chemical conversion of ethephon to ethylene for subsequent treatment of plants in a closed system has been tested (Zhang and Wen 2010). A kinetic analysis for the ethylene production with ethephon decomposition in the solution containing disodium phosphate (5 mM) as the reaction buffer showed that ethylene production was tightly correlated with the amount of ethephon, with $R^2 > 0.99$. Ethylene from a commercial gas tank and the ethylene produced by ethephon decomposition gave identical ethylene dose–response curves, when measuring hypocotyl length of etiolated *Arabidopsis* seedlings. Also, the purity of the ethylene produced from ethephon was examined by gas chromatography (GC), and no other hydrocarbon

species were detected. Thus, the ethylene produced from ethephon can be used directly for biological experiments. Of note, production of ethylene by ethephon decomposition is a cost-effective alternative to purchasing a compressed tank of ethylene. The decomposition of 8.3 μmole ethephon in a 2-L container produces an ethylene concentration of 102.83 ± 6.15 μL L^{-1} and 0.83 μmole ethephon an ethylene concentration of 9.47 ± 0.17 μL L^{-1}. Both ethylene concentrations are sufficient to saturate ethylene responses for most biological experiments.

For laboratories without the equipment to deliver the ethylene, the ethephon decomposition can occur in an airtight, closed chamber, along with the biological material to be treated, to produce ethylene without further gas handling. For example, in an experiment to determine the ethylene dose–response of etiolated *Arabidopsis* seedlings, ethylene introduced from a pressurized commercial tank and ethylene produced from ethephon decomposition produced nearly identical results (Zhang and Wen 2010; Zhang et al. 2010). For ethylene responses that require a short response window, the pH of the buffered solution needs to be higher than 9.0 to rapidly release ethylene and mixed with the ethephon while inside the closed chamber. This can be accomplished by injecting the buffer through a septum into a container of ethephon (see below). When the exact concentration of ethylene must be known, ethylene in the chamber can be quantified as described in Chap. 14.

13.4 Handling of the Ethylene Gas

Unlike many other plant growth substances that can be prepared in aqueous solutions, ethylene is a gas and its handling requires special equipment. Two main approaches for ethylene gas treatments are described: (1) a closed system where ethylene of a known concentration (e.g., 1,000 μL L^{-1}) is injected into a chamber of known volume (e.g., 1.0 L), and (2) a regulated concentration of ethylene in air is passed continuously through the chamber. Of note, in the dark, plants and plant organs consume oxygen in respiration and emit CO_2, water vapor, and other volatiles, and in the light the amount of oxygen produced and CO_2 consumed can vary. In other words, in a closed system, the concentration of gases can change, while in an open flow-through system, the gas concentrations are kept more constant.

13.4.1 Ethylene Treatment in a Closed System

For a closed system, the ethylene gas must be transferred from a source to an airtight chamber in which the plant material is placed. The gas concentration for ethylene is usually presented as μL L^{-1}, and, for most experiments, ethylene responses can be saturated within a concentration range of 1–10 μL L^{-1}. The gas chamber can be an airtight container of any type; a canning jar (Mason jars, with a band and lid) is ideal for small plant materials (such as seedlings, detached leaves

on *Petri* dishes, or small fruits) or a desiccator or custom-made acrylic chamber of for larger plant materials (such as plants in pots).

For most gas chambers, a rubber stopper can be sealed in the top or side of the chamber to create a gas inlet/outlet to facilitate the injection of the ethylene gas and the sampling of the chamber gas for ethylene and other volatile measurement. To create the gas inlet/outlet, a hole is drilled in the container, and a rubber stopper is pushed into or placed over the hole and sealed with silicone sealants. The rubber stopper can be of any kind; a re-sealable stopper is preferred because it can allow for repetitive injection and sampling of the gas, with a syringe and a needle, with little impact on airtightness. A variety of sleeve and vaccine stoppers can be used. A medical syringe needle with a bevel tip is not recommended for multiple sampling, because it can destroy the airtightness of the rubber stopper or the septa pinhole. We prefer to use the inlet septa for GC (part number: 5183-4761 from Agilent Technologies or its replacements) as the stopper and a GC manual syringe because the re-sealable pinhole at the septa can be repeatedly used with the GC syringe, of which the cone tip needle does not destroy the septa and will maintain airtightness.

The ethylene source can be a pressurized gas tank or a flask containing the ethylene gas at a known concentration. For most treatments, the amount of ethylene to be administered is very small. For instance, only 100 μL ethylene is needed for a 10 μL L^{-1} ethylene treatment in a 10 L chamber. Typically, the pure ethylene stock is diluted by injecting a known concentration of ethylene into a closed flask that you have measured the volume of by weighing the empty flask and then filing it to capacity with water and weighing again (water is 1.0 g mL^{-1}). It is essential that the flask be completely dry before making the gas dilution because ethylene is partially soluble in water. Often, if pure ethylene is used as a starting material, a series of dilutions will be required to sufficiently reduce the ethylene concentration.

The pressure in a pressurized tank is generally quite high, and taking the gas directly from the tank can be highly dangerous and technically difficult. A reducing valve (or pressure regulator) connected to the gas tank reduces the gas pressure to facilitate the safe use of the gas. A double-stage regulator is preferred because it has two valves. A rubber or silica tubing connected to the pressure regulator is used to collect pure ethylene from the tank. To do this, a clamp seals the end of the tubing and low-pressure ethylene is released from the tank, then the clamp is released to flush the air away. The flushing is repeated several times to ensure that the air in the tubing is flushed away and replaced by ethylene. Alternatively, the end of the tube can be sealed with a sleeve-type stopper with a small-gauge needle (#26) inserted in the stopper to continuously evacuate the gas for a few minutes before withdrawing pure ethylene from the tube. A medical syringe (preferably an insulin syringe) with a bevel tip needle can be used to withdraw the ethylene gas from the tubing.

Of note, an accurate concentration is difficult to achieve when a fairly small amount of gas is transferred for dilution, because a very small variation that occurs on the stock measuring will result in a large variation in the final concentration. For example, measuring 100 μL with a standard 1.0 mL insulin syringe is not particularly accurate. A larger volume is more accurately measured than a smaller one. For an ethylene dose–response assay, it is particularly useful to dilute the ethylene

stock by serial dilution. To improve accuracy of the dilutions, we use a constant volume of gas (e.g., 1.0 mL) when transferring the gas from the stock to each descending dilution. As long as the dilution flasks are completely sealed, the diluted ethylene stocks can be prepared in advance.

As mentioned previously, an alternative to a pressurized tank is preparation of ethylene by decomposing a necessary amount of ethephon with an alkaline solution in an airtight container. The airtight container could be a container separate from the treatment container, wherein ethylene is withdrawn and injected into the treatment container or the actual treatment container in which the alkaline solution is injected into a vial containing the appropriate amount of ethephon. If necessary, the concentration of ethylene can be confirmed by gas chromatography. We showed that the commercial ethylene and chemically prepared ethylene have identical biological effect on the growth of *Arabidopsis* seedling and induction of the ethylene-inducible *ERF1* gene (Zhang and Wen 2010; Zhang et al. 2010).

The setup of ethylene treatment in a closed system is simple and cost-effective and can be versatile with the use of nearly any airtight container with a gas septa inserted or sealed onto the chamber. Here we describe two types of containers that have been tested for ethylene treatment in a closed system (Fig. 13.1).

For treating small plant materials, such as *Arabidopsis* seedlings and detached leaves on a *Petri* dish, any airtight container with a diameter greater than that of a *Petri* dish will suffice. The volume of the *Petri* dish (including the growth medium) can be estimated. Because the amount of ethylene that partitions into aqueous solutions (media) is small compared to the amount in the gas space, the volume the *Petri* with media can be deducted from container volume. For instance, if the net gas volume of the container is 0.5 L and the ethylene stock is 1,000 μL L^{-1}, 0.5 mL of the ethylene stock is injected to achieve a final ethylene concentration of 1 μL L^{-1}. To ensure airtightness, we usually seal the "mating surfaces" on the lid and the container with grease (such as Vaseline) to prevent gas leakage. The gas inside the container can be sampled periodically and quantified by gas chromatography to determine changes in the amount of ethylene in the container. For ethylene treatment of larger plant materials, such as *Arabidopsis* rosettes or rice (*Oryza sativa*) seedlings, we use a custom-made acrylic container. The "mating surface" of the chamber is sealed with a flat rubber gasket to ensure airtightness when lids are closed. Spring toggle latches (spring-loaded latch) on each side of the chamber secure the lid. A hole is drilled on the lid and a piece of gas septa is attached to the hole and sealed by silicone sealant. The container volume can be calculated by the chamber size, and the volume for the plant material (i.e., the pot volume) can be estimated and deducted from the chamber volume. An appropriate amount of ethylene stock is injected and can be quantified as described above. We do not recommend the use of laboratory glassware (such as a test tube or an Erlenmeyer flask) coupled with a matching rubber stopper for ethylene treatment. The stopper may slip over time without being noticed, and the slipping may result in gas leakage.

Precautions should be noted for ethylene treatment in a closed system. Plants emit water and the humidity in a closed chamber/container may increase throughout

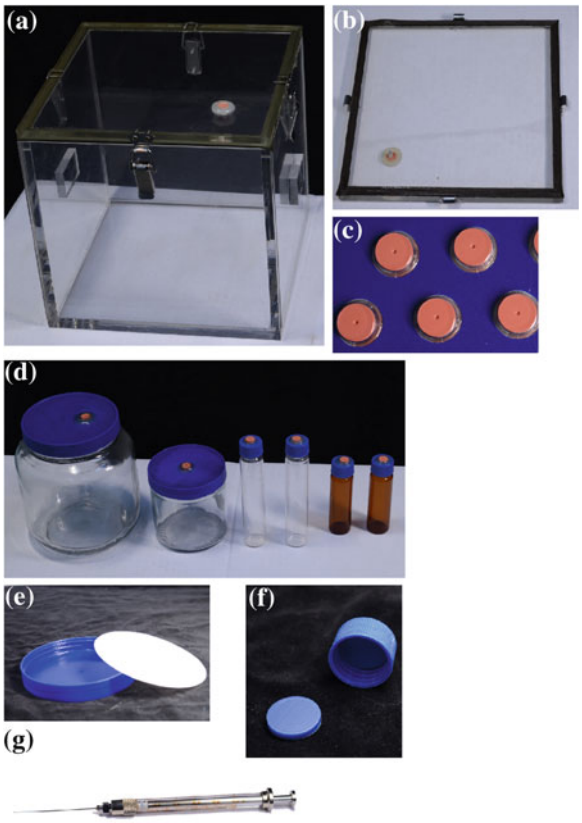

Fig. 13.1 Setup for ethylene treatment in an airtight closed system. **a** An airtight acrylic container for ethylene treatment in a closed system. Spring toggle latches on each side of the chamber fasten the lid. **b** On the lid, a hole is sealed with a gas chromatography (GC) inlet septa (*red*) that has a re-sealable pinhole for repeated gas delivery and sampling and a flat rubber gasket (*dark*) on the mating surface to ensure airtightness. **c** The GC septa inlet has a re-sealable pinhole. **d** An example of a container of other types that can be used for ethylene treatment. **e**, **f** A gasket or pad on the matching surface inside the lid of a container to ensure airtightness. **g** A GC syringe with a cone tip needle to deliver the gas

the entire treatment when the water content in the soil is high. As a result, water will condense and accumulate on the leaves to produce unwanted effects to the plant material. To prevent this situation from happening, absorbing paper is placed underneath the pot to absorb excess water, and the soil water content is kept at a low level sufficient for normal plant growth at the beginning of the treatment. Given that plants produce the ethylene gas, the ethylene concentration will also increase over time in a closed chamber. To minimize ethylene concentration fluctuation, a container or chamber of a large volume is preferred so that the emitted ethylene has less impact on the final ethylene concentration.

13.4.2 Flow-Through System for Ethylene Treatment

In a closed system, the concentration of ethylene may change slightly over time. Typically, the ethylene concentration will increase because the plant synthesizes ethylene. A marked decline in ethylene usually indicates a small leak in the system. However, if the duration of the experiment is fairly long, several hours to days, the change in CO_2, oxygen, and other volatiles can have marked effects on plant growth and the plants response to ethylene. When a more constant level of ethylene, oxygen, and CO_2 concentration is required, a flow-through system can greatly reduce variation in the composition of the gas environment. A fairly simple and inexpensive setup for doing this is in Fig. 13.2.

The central component in a flow-through system is a chamber that can be sealed except for a gas inlet and outlet. A vacuum desiccator can be used for this purpose. Some chambers come with two ports and others will need a port added. It is worth mentioning that, even though most chambers come with good quality latches, in some chambers it is difficult to achieve a 100 % seal on the door when no vacuum is being applied. However, in a flow-through system, a small leak less than 10 % the outlet flow can be tolerated because these leaks will have a negligible effect on the concentration of ethylene and other gases inside the chamber. If gas sampling is required to confirm the ethylene concentration inside the chamber, a silicone hose can be connected to the outlet and a gas sample withdrawn. The gas sample must be withdrawn slowly so as not to pull outside air into the open end of the tube and into the syringe.

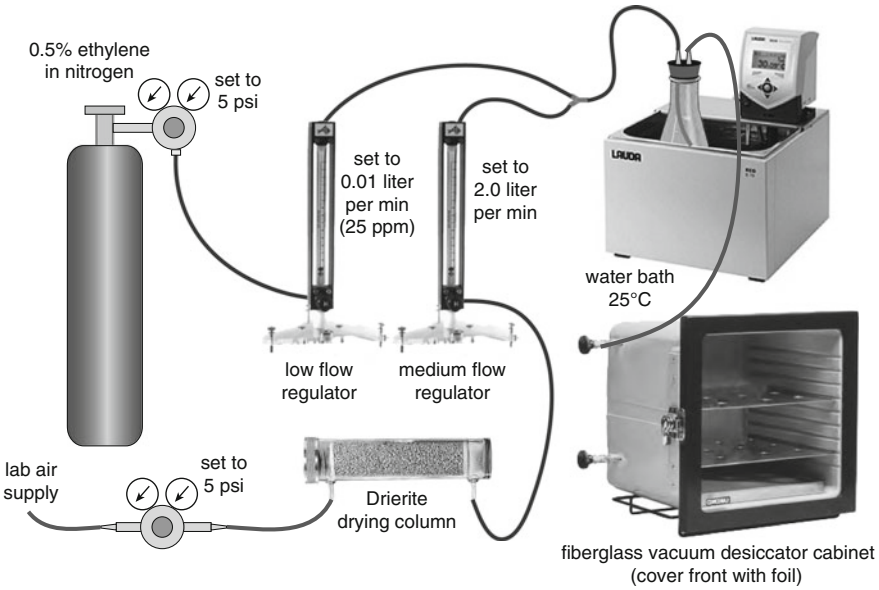

Fig. 13.2 Setup for flow-through ethylene treatment of plant material

Light and diurnal circadian rhythms affect most ethylene responses and need to be addressed. We use an opaque fiberglass chamber with a clear Plexiglas door, which has an approximate volume of 33 L. The clear door must be covered with aluminum foil or black paper to maintain darkness. We collect plant tissue at the same time each day (e.g., morning), and place the tissue in the dark chamber for ethylene treatment. However, for a light–dark cycle, a clear Plexiglas vacuum desiccator chamber can be obtained and kept in a lighted incubator.

Also important to any biological system is maintenance of temperature. The chamber can be kept inside a constant temperature room or incubator, but the gas mixture may be passed through a sealed flask held in a temperature-regulated circulating water bath to bring all the gases to the desired temperature before entering the sealed chamber (Fig. 13.1). If the chamber is kept in a room where the temperature is relatively close to the desired temperature, passing the gas mixture through the flask is sufficient to maintain a constant temperature in the chamber. We use a 2 L Erlenmeyer flask containing approximately 100 mL distilled water. The flask is sealed with a rubber stopper. Small holes are bored through the stopper and plastic inlet, and outlet tubes are pushed through the holes to make an airtight fit. To improve temperature equilibration and humidify the gas, Tygon tubing is connected to the inlet on the inside of the flask so that the tube extends down to the surface of the water in the bottom of the flask. The gas exits the flask through an outlet that extends only a short distance into the flask.

Preparing the gas mixture requires an air supply and a source of a high concentration of ethylene. We use the building air supply for our air source, but an inexpensive pump that can deliver 2 L min^{-1} with an outlet pressure of 5 psi or greater will suffice. The pressure of our lab air supply varies. We use a single-stage gas regulator to maintain a constant pressure of 5 psi at the outlet. We pass lab air through an approximately 0.5 L acrylic Drierite gas drying column (DRIERITE cat no. 26800), which removes moisture but also acts as a rough filter. Condensation must be prevented in the flow regulators that follow the Drierite column. Condensation will clog the regulators and change the flow rate. Most lab air has a low level of ethylene in it. Generally this can be ignored. However, if necessary for a control treatment, the air supply can be scrubbed of ethylene and other hydrocarbons by adding a second gas column packed with Purafil Select Media (a potassium permanganate product).

For our ethylene source, we use a 2,000 psi 44 L tank of 0.5 % ethylene in nitrogen (specialty gas mixture from Airgas). A different concentration can be used, but this works well with our flow regulators, flow rate, and final concentration. A tank of this size and concentration will last for quite a long time. A two-stage nitrogen gas regulator is required to reduce the outlet pressure to 5 psi. These gas mixtures are already dry and do not usually require a drying column.

The flow rate of the air and ethylene gases must be regulated and mixed to obtain an ethylene concentration in air of between 5 and 25 μL L^{-1}. In most plant systems, 0.1 μL L^{-1} ethylene in air produces a half-maximal ethylene response (Abeles et al. 1992). A concentration of 10 μL L^{-1} is commonly used as a saturating concentration to produce a maximal ethylene response (Abeles et al. 1992; Lincoln and

Fischer 1988); however, to insure a more rapid response for shorter time intervals, we use 25 μL L^{-1} ethylene in air with a final flow rate of 2.0 L min^{-1}. To obtain this concentration and flow rate, we use two rotameters (gas flow meters). A variety of flow meters can be used for this task. We use a flow meter that uses a 150 mm flow tube. These devices are fitted with different-sized tubes and valves to achieve the required flow rates. One of the flow meters should accurately (±5 %) measure an airflow between 0.5 and 5.0 L min^{-1} (LPM). The other flow meter should measure an air (nitrogen) flow between 0.002 and 0.050 LPM. This will dilute the 0.5 % ethylene in nitrogen (5,000 μL L^{-1}) with air to achieve an ethylene concentration between 5 and 25 μL L^{-1}. In our setup, for the air supply, we use a flow tube FM4331 with a standard metering valve 0202-4113 (L) from Specialty Gas Equipment (ASGE). The low-flow meter for the ethylene gas is fitted with a flow tube FM4334 with a standard metering valve 0202-4114 (M). After metering, the two flows are then combined before entering the Erlenmeyer flask in the water bath and then the chamber. We have successfully used this system for many years (Kalaitzis et al. 1995; Tucker et al. 1988; Tucker and Yang 2012).

13.5 Concluding Remarks

As a plant hormone important to many aspects of plant growth and development, by itself and in combination with other hormones and biotic and abiotic cues, ethylene or its replacements are widely used for biological experiments to address the aforementioned phenomena and underlying mechanisms. For simplicity and short duration experiment, a closed system works well for ethylene treatments; for longer exposures to ethylene, a flow-through system is superior to a closed system. When pressurized gas cylinder is not conveniently available, replacements for ethylene can be used instead. Small amounts of ethylene can be generated efficiently and inexpensively by chemical conversion of ethephon to ethylene in an alkaline solution. In a closed system, the ethylene gas produced from ethephon works as well as ethylene from a pressurized gas cylinder. Alternatively, ACC and ethephon can be applied directly to the plant in media or a spray. However, precautions need to be considered for the use of the ethylene replacement: (1) aqueous ethephon solutions are of low pH and the ethephon decomposition to produce ethylene produces acid species, which may lead to unwanted effects that complicate data interpretation; (2) the conversion of ACC to ethylene is an oxidation reaction and a sufficient oxygen supply will ensure the reaction to proceed; (3) ACC can be alternatively converted to MACC, which cannot be converted to ethylene; (4) results from ACC application by foliar spray may be less reproducible because of early ACC consumption and unequal spraying; (5) treatment with ACC or ethephon to replace ethylene treatment is not ideal for reactions that require a long response window; and (6) lower plants produce ethylene via unidentified pathway(s) that do not use ACC as a precursor, so ACC is not an ideal replacement for ethylene treatment for lower plants.

Acknowledgments This work was supported by the National Natural Sciences Foundation of China (31123006 and 30770199) to CK Wen.

References

Abeles FB, Morgan PW, Salveit ME. Ethylene in plant biology. 2nd ed. New York: Academic Press; 1992.

Adams DO, Yang SF. Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc Natl Acad Sci. 1979;76(1):170–4.

Biddle E, Kerfoot DGS, Kho YH, Russell KE. Kinetic studies of the thermal decomposition of 2-chloroethylphosphonic acid in aqueous solution. Plant Physiol. 1976;58(5):700–2. doi:10.1104/pp.58.5.700.

Chernys J, Kende H. Ethylene biosynthesis in *Regnellidium diphyllum* and *Marsilea quadrifolia*. Planta. 1996;200(1):113–8. doi:10.1007/BF00196657.

Dahnous K, Vigue GT, Law AG, Konzak CF, Miller DG. Height and yield response of selected wheat, barley, and triticale cultivars to ethephon1. Agron J. 1982;74(3):580–2. doi:10.2134/agronj1982.00021962007400030041x.

Edwards ME. Carbon dioxide and ethylene control of spore germination in *Onoclea sensibilis* L. Plant Physiol. 1977;59(4):756–8. doi:10.1104/pp.59.4.756.

Fan D, Dai D-J, Wu H-S. Ethylene formation by catalytic dehydration of ethanol with industrial considerations. Materials. 2012;6(1):101–15.

Finlayson SA, Foster KR, Reid DM. Transport and metabolism of 1-aminocyclopropane-1-carboxylic acid in sunflower (*Helianthus annuus* L.) seedlings. Plant Physiol. 1991;96(4):1360–7. doi:10.1104/pp.96.4.1360.

Fisher RW, Shropshire W. Reversal by light of ethylene-induced inhibition of spore germination in the sensitive fern *Onoclea sensibilis*: an action spectrum. Plant Physiol. 1979;63(6):984–8. doi:10.1104/pp.63.6.984.

Goudey JS, Saini HS, Spencer MS. Uptake and fate of ethephon ([2-chloroethyl]phosphonic acid) in dormant weed seeds. Plant Physiol. 1987;85(1):155–7. doi:10.1104/pp.85.1.155.

Hoffman NE, Fu J-R, Yang SF. Identification and metabolism of 1-(Malonylamino)cyclopropane-1-carboxylic acid in germinating peanut seeds. Plant Physiol. 1983;71(1):197–9. doi:10.1104/pp.71.1.197.

Kalaitzis P, Koehler SM, Tucker ML. Cloning of a tomato polygalacturonase expressed in abscission. Plant Mol Biol. 1995;28(4):647–56.

Kende H. Ethylene biosynthesis. Ann Rev Plant Physiol Plant Mol Biol. 1993;44(1):283–307. doi:10.1146/annurev.pp.44.060193.001435.

Lavee S, Martin GC. Ethylene evolution following treatment with 1-aminocyclopropane-1-carboxylic acid and ethephon in an in Vitro olive shoot system in relation to Leaf abscission. Plant Physiol. 1981;67(6):1204–7. doi:10.1104/pp.67.6.1204.

Li Y, Solomon S. Ethephon: a versatile growth regulator for sugar cane industry. Sugar Tech. 2003;5(4):213–23.

Lincoln JE, Fischer RL. Regulation of gene expression by ethylene in wild-type and rin tomato (lycopersicon esculentum) fruit. Plant Physiol. 1988;88(2):370–4.

Martin MN, Cohen JD, Saftner RA. A new 1-aminocyclopropane-1-carboxylic acid-conjugating activity in tomato fruit. Plant Physiol. 1995;109(3):917–26. doi:10.1104/pp.109.3.917.

Martin MN, Saftner RA. Purification and characterization of 1-aminocyclopropane-1-carboxylic acid N-malonyltransferase from tomato fruit. Plant Physiol. 1995;108(3):1241–9. doi:10.1104/pp.108.3.1241.

Miller JM, Conn EE. Metabolism of hydrogen cyanide by higher plants. Plant Physiol. 1980;65(6):1199–202. doi:10.1104/pp.65.6.1199.

Murphy LJ, Robertson KN, Harroun SG, Brosseau CL, Werner-Zwanziger U, Moilanen J, Tuononen HM, Clyburne JAC. A simple complex on the verge of breakdown: isolation of the elusive cyanoformate ion. Science. 2014;344(6179):75–8. doi:10.1126/science.1250808.

Norberg OS, Mason SC, Lowry SR. Ethephon influence on harvestable yield, grain quality, and lodging of corn. Agron J. 1988;80(5):768–72. doi:10.2134/agronj1988.00021962008000050015x.

Osborne DJ, Norville A, Walters J, Milborrow BV, Stange LMC. Evidence for a non-ACC ethylene biosynthesis pathway in lower plants. Phytochemistry. 1996;42(1):51–60. doi:10.1016/0031-9422(96)00032-5.

Peiser G, Fa Yang S. Evidence for 1-(Malonylamino)cyclopropane-1-carboxylic acid being the major conjugate of aminocyclopropane-1-carboxylic acid in tomato fruit. Plant Physiol. 1998;116(4):1527–32. doi:10.1104/pp.116.4.1527.

Peiser GD, Wang T-T, Hoffman NE, Yang SF, Liu H-w, Walsh CT. Formation of cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to ethylene. Proc Natl Acad Sci. 1984;81(10):3059–63.

Pommerrenig B, Feussner K, Zierer W, Rabinovych V, Klebl F, Feussner I, Sauer N. Phloem-specific expression of yang cycle genes and identification of novel yang cycle enzymes in plantago and arabidopsis. Plant Cell Online. 2011;23(5):1904–19. doi:10.1105/tpc.110.079657.

Reid MS, Paul JL, Young RE. Effects of pH and ethephon on betacyanin leakage from beet root discs. Plant Physiol. 1980;66(5):1015–6. doi:10.1104/pp.66.5.1015.

Seo S, Mitsuhara I, Feng J, Iwai T, Hasegawa M, Ohashi Y. Cyanide, a coproduct of plant hormone ethylene biosynthesis, contributes to the resistance of rice to blast fungus. Plant Physiol. 2011;155(1):502–14. doi:10.1104/pp.110.162412.

Solano R, Stepanova A, Chao Q, Ecker J. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. 1998;12:3703–14.

Southwick KL, Lamb N, Storey R, Mansfield DH. Effects of ethephon and its decomposition products on germination of rice and watergrass1. Crop Sci. 1986;26(4):761–7. doi:10.2135/cropsci1986.0011183X002600040028x.

Takahara I, Saito M, Inaba M, Murata K. Dehydration of ethanol into ethylene over solid acid catalysts. Catal Lett. 2005;105(3–4):249–52. doi:10.1007/s10562-005-8698-1.

Tucker ML, Sexton R, del Campillo E, Lewis LN. Bean abscission cellulase: characterization of a cDNA clone and regulation of gene expression by ethylene and auxin. Plant Physiol. 1988;88:1257–62.

Tucker ML, Yang R. IDA-like gene expression in soybean and tomato leaf abscission and requirement for a diffusible stelar abscission signal. AoB plants 2012:pls035. 2012. doi:10.1093/aobpla/pls035.

Yang S-F. Ethylene evolution from 2-chloroethylphosphonic acd. Plant Physiol. 1969;44:1203–4.

Yoon GM, Kieber JJ. 1-aminocyclopropane-1-carboxylic acid as a signalling molecule in plants. AoB Plants 5. 2013. doi:10.1093/aobpla/plt017.

Zhang M, Yu Y. Dehydration of ethanol to ethylene. Ind Eng Chem Res. 2013;52(28):9505–14. doi:10.1021/ie401157c.

Zhang W, Hu W, Wen C-K. Ethylene preparation and its application to physiological experiments. Plant Signal Behav. 2010;5(4):453–7.

Zhang W, Wen C-K. Preparation of ethylene gas and comparison of ethylene responses induced by ethylene, ACC, and ethephon. Plant Physiol Biochem. 2010;48(1):45–53.